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Kartic Padmanabhan
Patent Examiner
Art Unit 1641
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W.D.

Antibodies to E1 and E2/Protein X components of pyruvate dehydrogenase complex in sera of patients with primary biliary cirrhosis

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Aims/Methods: Using purified E1 component of pyruvate dehydrogenase complex (PDC) from bovine heart, we measured the levels of anti-E1 antibodies in PBC sera using ELISA and determined the degree of inhibition that these antibodies exerted on E1 enzyme activity. We also estimated levels of anti-E2/Protein X (Pro-X) antibodies in PBC sera using purified E2 and Pro-X of PDC which were copurified with E1.

Results/Conclusions: Anti-E1 antibodies were detected in 87.5% (35/40) of PBC sera. Some of these sera inhibited E1 enzyme activity but inhibition did not correlate with levels of anti-E1 antibodies.

A high positive correlation ($r=0.918$) was found between levels of anti-E1 and anti-E2/Pro-X antibodies, suggesting that anti-PDC antibody production was stimulated by PDC itself. Levels of IgG class anti-E2/Pro-X antibodies were significantly higher in sera of symptomatic PBC patients than in those of asymptomatic PBC patients. It was also found that patients who were positive for only IgM class anti-E2/Pro-X antibodies had early-stage PBC.

Key words: Anti-E1 antibody; Anti-E2/Pro-X antibody; Anti-PDC antibody; Primary biliary cirrhosis.

ANTI-MITOCHONDRIAL antibodies (AMA) have been recognized as markers of PBC, and M2 antigens are known as antigens that react with AMA in sera of PBC patients. Recently, these antigens have been revealed to be components of enzyme complexes such as the pyruvate dehydrogenase complex (PDC) (1,2), the branched-chain α -ketoacid dehydrogenase complex (3) and the α -ketoglutarate dehydrogenase complex (α -KGDH) (4). PDC is a multi-enzyme complex which consists of five components: E2, Protein X (Pro-X), E3, E1- α and E1- β . Of these, the E2 component of PDC has been recognized as the major autoantigen among M2 antigens (4).

It was recently shown in an experiment with AMA that Pro-X possesses epitopes which are cross-reactive with PDC-E2 (5). It was also reported that autoantibodies to PDC-E2/Pro-X are present in most patients (93%) with PBC, and that the titer of IgG class anti-E2/Pro-X antibody correlated with the histological stage of PBC and with the serum levels of bilirubin and albumin (6). Moreover, Fregeau et al. (7) reported that autoantibody to PDC-E1 was detected in 66% of PBC sera by immunoblotting analysis, and that affinity-purified PBC sera which reacted with PDC-E1- α inhibited PDC overall activity. However, no study has investigated the correlation between levels of antibodies to E1 or to E2/Pro-X and clinical symptoms. There is also no report on the effect of PBC sera on the enzyme activity of E1 component itself. In this study, we purified the E1 and E2/Pro-X components of PDC from bovine heart and investigated the correlation between levels of the antibodies to E1 or E2/Pro-X components of PDC and clinical symptoms. We further analyzed the effect of PBC sera on E1 enzyme activity and compared that with levels of anti-E1 antibodies.

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Materials and Methods

Patients and sera

Sera were collected from 40 PBC patients (17 symptomatic (s) and 23 asymptomatic (a)), all with a well established diagnosis based on clinical and laboratory findings (8). Seventeen patients with s-PBC showed icterus or pruritus. Twenty-three patients were found to have a-PBC because of raised alkaline phosphatase levels and were subsequently diagnosed by liver biopsy. Histologically, patients were classified into stages I/II (24 cases) or stages III/IV (16 cases) according to the criteria of Scheuer (9). Thirty-five sera reacted on indirect immunofluorescence with mitochondria on frozen sections of rat kidney at titers ranging from 1:80 to 1:640. AMA was not detected in five cases. Levels of serum IgM were elevated in all cases and values of total serum bilirubin were greater than 2 mg/dl in 11 cases. In 18 cases, PBC was complicated by the presence of other autoimmune diseases: Sjögren syndrome in 15, CREST syndrome in two and autoimmune thyroiditis in one. Diagnosis of Sjögren syndrome was made using the criteria of the European Community (10); five of these cases were defined as definite while ten were considered probable. Anti-SSA antibody was positive in sera of three of the definite cases whereas anti-SSB antibody was positive in none. Controls consisted of 20 patients with acute viral hepatitis (AVH, ten type A and ten type B), 20 with chronic viral hepatitis (CH, ten type B and ten type C), 20 with autoimmune hepatitis (AIH), 20 with liver cirrhosis (LC, ten type B and ten type C) and 20 healthy subjects (HS). A diagnosis of AIH was made using the criteria proposed by the International Autoimmune Hepatitis Group (11). Blood was taken from the cubital vein and sera were prepared by clotting the specimen and storing the sera at -20°C until use. Patients were mostly inpatients of our department. Healthy subjects were selected from among staff members.

Purification of PDC

PDC was purified according to the method of Stanley & Perham (12). Briefly, frozen ox hearts were suspended in 50 mM Mops (4-morpholinepropanesulphonic acid), pH 7.0, containing 2.7 mM EDTA, 0.1 mM dithiothreitol and 3% (v/v) Triton X-100, and blended in a Waring blender. The homogenate was then centrifuged by a differential centrifugation-precipitation method using 35% polyethylene glycol. The precipitate was resuspended in 50 mM Mops buffer and centrifuged. The supernatant was collected, its pH adjusted to 5.4 by the addition of 10% acetic acid, and it was then centrifuged. The superna-

tant obtained contained more than 95% of PDC activity and less than 5% of α -KGDH activity, as determined by spectrophotometric measurement, monitoring NADH production (12). The supernatant was then subjected to gel filtration using a Sepharose CL-2B column (Pharmacia Biotech, Uppsala, Sweden) and the PDC-active and α -KGDH-inactive fractions (260–320 ml), in which protein concentrations were proportional to PDC activity, were collected (sample A). The SDS-PAGE of sample A gave five bands representing proteins with MWs of 74 kD, 55 kD, 50 kD, 42 kD and 37 kD, which corresponded to E2, E3, Pro-X, E1- α and E1- β components of PDC, respectively, based on previously published information on ox heart enzymes (13).

Isolation of E1 and E2/Pro-X from PDC

Purified PDC was further dissolved in 0.1 M glycine buffer containing 1 M NaCl at room temperature for 30 min (14) and then applied to a Sepharose CL-4B column (Pharmacia Biotech, Uppsala, Sweden). Two protein peaks were obtained: the earlier peak contained 74 and 50 kD proteins and the second peak, 55, 42 and 37 kD proteins, as estimated by SDS-PAGE. Thus, the former peak (sample B) contained E2 and Pro-X and the latter, E3, E1- α and E1- β . Fractions representing the latter peak were pooled and applied to a hydroxyapatite column and the retained proteins were eluted with 0.35 M potassium phosphate buffer (KPB) (designated sample C) or with 0.2 M KPB (designated sample D).

The above procedures were performed at 4°C except for resolution of PDC.

SDS-PAGE and immunoblotting

SDS-PAGE was performed on 1.5-mm-thick slab gels with a 5% stacking gel and a 10% resolving gel (15). Approximately 5–10 μg of protein samples were loaded in each lane and run at 30 mA at room temperature. Proteins were either stained with Coomassie brilliant blue R or transferred electrophoretically to nitrocellulose sheets at 200 mA for 90 min.

Immunoblotting (16) was performed at room temperature and all dilutions were made with PBS containing 0.05% Tween-20 (PBS-Tween). The nitrocellulose sheets were treated for 1 h at room temperature with 3% BSA in PBS and probed for 1.5 h with the PBC serum diluted to 1/1000. Sheets were washed five times with PBS-Tween and incubated at room temperature for 1 h with ALP-conjugated goat anti-human IgG (all) antibodies (Tago Inc., Burlingame, USA) diluted to 1/1000. After washing five

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times with PBS-Tween, color was developed by plac-
ing the membrane in a solution of naphthol FAS
phosphate and fast blue BB salt (Sigma Chemical
Co., St. Louis, MO, USA).

Enzyme-linked immunosorbent assay (ELISA) for detection of anti-E1 and anti-E2/Pro-X antibodies

One hundred microliters of the sample containing
1 μ g of either purified E1 (fraction of hydroxyapatite
column) or purified E2/Pro-X (earlier fraction of the
CL-4B column) were placed in each well of a micro-
titer plate and left overnight at 4°C. After washing
three times with PBS, wells were filled with 1%
BSA-PBS for postcoating and incubated at room tem-
perature for 1 h. Wells were washed three times with
PBS-Tween, treated with 100 μ l of test sera diluted to
1/1000 and the plate was left at room temperature for
1 h. Wells were then washed as above and 100 μ l of
ALP-conjugated anti-human IgG, IgM, IgA antibody
or anti-human immunoglobulin antibody, diluted to
1/2000, 1/1000, 1/1000 or 1/2000 with PBS-Tween,
respectively, were added to each well and incubated at
room temperature for 30 min. The wells were washed
five times with PBS-Tween, and 100 μ l of p-nitro-
phenyl phosphate (1 mg/ml, Sigma) in 0.05 M car-
bonate-bicarbonate buffer (substrate buffer), pH 9.8,
containing 0.01 M $MgCl_2$ were added to each well.
After incubation for 15 min at room temperature, ODs
were measured using a double-beam spectrophoto-
meter (Bio-Rad, USA) at a wavelength of 405nm.

Assay of E1 activity

The enzyme activity of E1 was assayed by monitoring
 $^{14}CO_2$ production with 1- ^{14}C pyruvic acid as the sub-
strate (17). A total 1 ml volume of the assay mixture
contained 400 mM KPB, 0.6 mM thiamine pyrophos-
phate, 1 mM $MgCl_2$, 0.5 mM 2,6-dichloroindophenol,
5 mM 1- ^{14}C pyruvic acid (13.4 MBq/mg, Amersham
International, UK) and the E1 preparation. After prein-
cubation for 20 min, the reaction was initiated by the
addition of the labeled substrate and allowed to pro-
ceed for 20 min at 35°C. The reaction was terminated
by the addition of 50 μ l of 50% TCA and the $^{14}CO_2$
formed was absorbed by Scintillamin-OH. Radioactiv-
ity was measured using a scintillation counter. To
measure the rate of inhibition of E1 by PBC sera, 100
 μ l of the undiluted sera were added immediately prior
to the addition of the labeled substrate.

Statistical analysis

The results were expressed as means \pm SD. Differ-
ences were examined using Welch's t-test and a value
of $p < 0.05$ was considered significant.

Results

SDS-PAGE analysis of PDC preparations and com- ponents

Samples A (Sephacose CL-2B preparation), B (earlier
fraction of Sephacose CL-4B), C (fraction from
hydroxyapatite column using 0.35 M KPB) and D

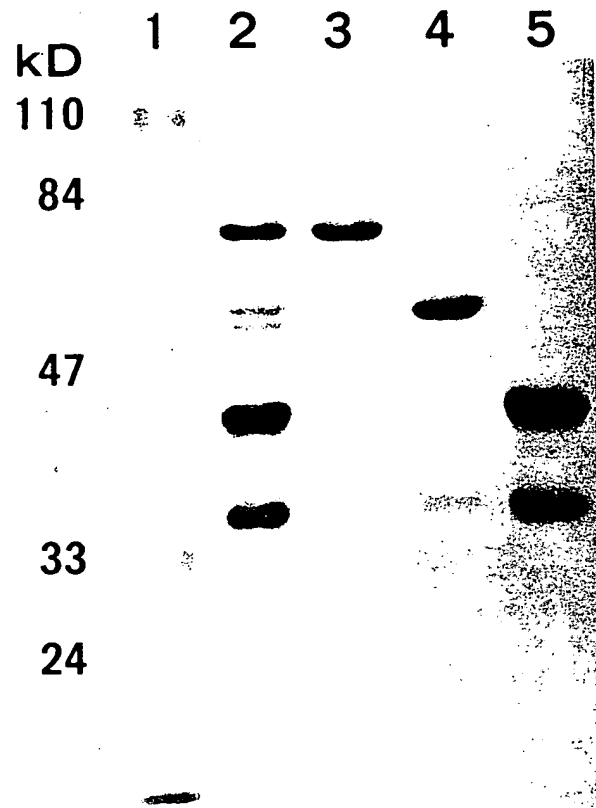


Fig. 1. SDS-PAGE analysis of PDC preparations. Four
preparations were applied using a 5% stacking gel and
10% resolving gel. Materials were run at 30 mA at room
temperature and stained with Coomassie brilliant blue R.
Lane 1, Marker proteins (10 μ l/lane). Lane 2, Sample A
(CL-2B column preparation, 10 μ g/lane). Lane 3, Sample
B (early fraction of CL-4B preparation, 5 μ g/lane). Lane
4, Sample C (0.35 M KPB preparation from hydroxyapatite
column, 5 μ g/lane). Lane 5, Sample D (0.2 M KPB prepa-
ration from hydroxyapatite column, 5 μ g/lane).

Sample A showed five bands of 74 kD (E2), 55 kD (E3), 50
kD (Protein X), 42 kD (E1- α) and 37 kD (E1- β). Sample B
showed two bands of 74 kD (E2) and 50 kD (Protein X).
Sample C showed a strong band of 55 kD (E3) and two
faint bands of 42 kD (E1- α) and 37 kD (E1- β). Sample D
showed two strong bands of 42 kD (E1- α) and 37 kD
(E1- β). Thus, samples A, B and D contained all compo-
nents of PDC, purified E2/Pro-X and purified E1, respec-
tively, whereas sample C contained E3 and a small amount
of E1.

(fraction from hydroxyapatite column using 0.2 M KPB) were analyzed on SDS-PAGE. As seen Fig. 1, sample A (lane 2) showed five bands of 74 kD, 55 kD, 50 kD, 42 kD and 37 kD. These molecular weights were consistent with those of E2, E3, Pro-X, E1- α and E1- β components of PDC, as previously reported for ox heart enzymes, respectively (8,9). Sample A, therefore, was assumed to be purified PDC. Sample B (lane 3) showed two bands of 74 kD (E2) and 50 kD (Pro-X). Sample C (lane 4) showed one strong band of 55 kD (E3) and two other faint bands of 42 kD (E1- α) and 37 kD (E1- β). Sample D (lane 5) showed two strong bands of 42 kD (E1- α) and 37 kD (E1- β). Thus, sample D was assumed to be purified E1.

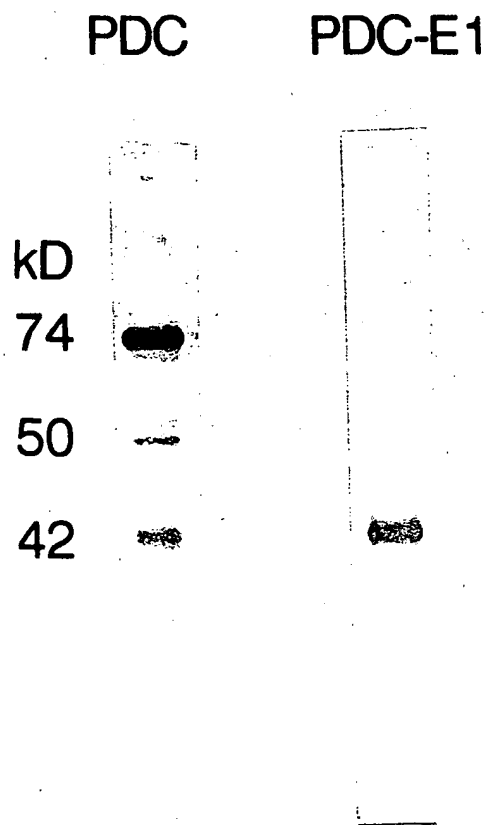


Fig. 2. Immunoblotting of purified PDC or purified E1 with PBC serum. PBC serum (K.E.) diluted at 1/1000 showed three main bands of 74 kD (E2), 50 kD (Protein X) and 42 kD (E1- α) when probed against purified PDC as seen on the left. The same serum diluted to 1/200 reacted with purified E1, showing a definite band of 42 kD (E1- α), as seen on the right.

Immunoblotting and ELISA for anti-E1 and anti-E2/Pro-X antibodies in sera of patients with various liver diseases

The purified PDC preparation was applied to SDS-PAGE. Separated proteins were transferred to a nitrocellulose sheet which was then treated with 1/1000 diluted PBC serum (K.E.). Three main bands of 74 kD, 50 kD and 42 kD, corresponding to E2, Pro-X and E1- α , respectively, can be seen in the left lane in Fig. 2. When the same serum (K.E.) diluted to 1/200 was analyzed with sample D (purified E1) instead of the purified PDC preparation, one band was observed at 42 kD (E1- α), as seen in the right lane in Fig. 2, indicating that sample D did not contain the E2/Pro-X components which affected the OD values of anti-E1 antibody in ELISA using this E1 antigen. Next, 40 sera of PBC patients, 20 sera of other liver disease patients and 20 sera of healthy subjects were similarly tested by immunoblotting. The results obtained are summarized in Table 1. Thirty-six of 40 (90%) PBC sera showed a positive reaction with the 74 kD (E2) and 50 kD (Pro-X) components of PDC. Twenty-five of 40 (62.5%) PBC sera that were positive for the 42 kD (E1- α) components reacted with 74 kD (E2) and 50 kD (Pro-X) whereas 11 sera reacted only with the 74 kD (E2) and 50 kD (Pro-X) components. There were no sera that reacted with either E1 or E3 alone. In addition, none of the 20 sera from the patients with other liver diseases or those from healthy subjects reacted with these antigens.

Results of ELISA for anti-E1 and anti-E2/Pro-X antibodies are shown in Fig. 3. Before performing the experiment, we carried out an absorption test to confirm the specificity of the ELISA system. The OD value obtained with an anti-E1 positive serum did not decrease with the addition of purified E2/Pro-X to the serum before treating with E1 but it did decrease with the addition of purified E1 and vice versa (data not shown). Results of ELISA for anti-E1 antibodies are shown on the left side in Fig. 3. Positive values that were +4SD higher than the mean value of sera from healthy subjects were observed only with sera of PBC patients. Positive reactions were obtained with 87.5% (35/40) of PBC sera, whereas none were obtained with 100 sera of patients with other liver diseases or healthy subjects. The mean OD value of anti-E1 antibodies in PBC sera was 0.509 ± 0.307 , as shown in Fig. 3. Similarly, values of anti-E2/Pro-X antibodies from ELISA are shown on the right side in Fig. 3. Positive values that were +4SD higher than the mean value of healthy subjects were observed only with sera of PBC patients (37/40, 92.5%). Mean OD val-

anti-E1 antibody

anti-E2 / Pro-X antibody

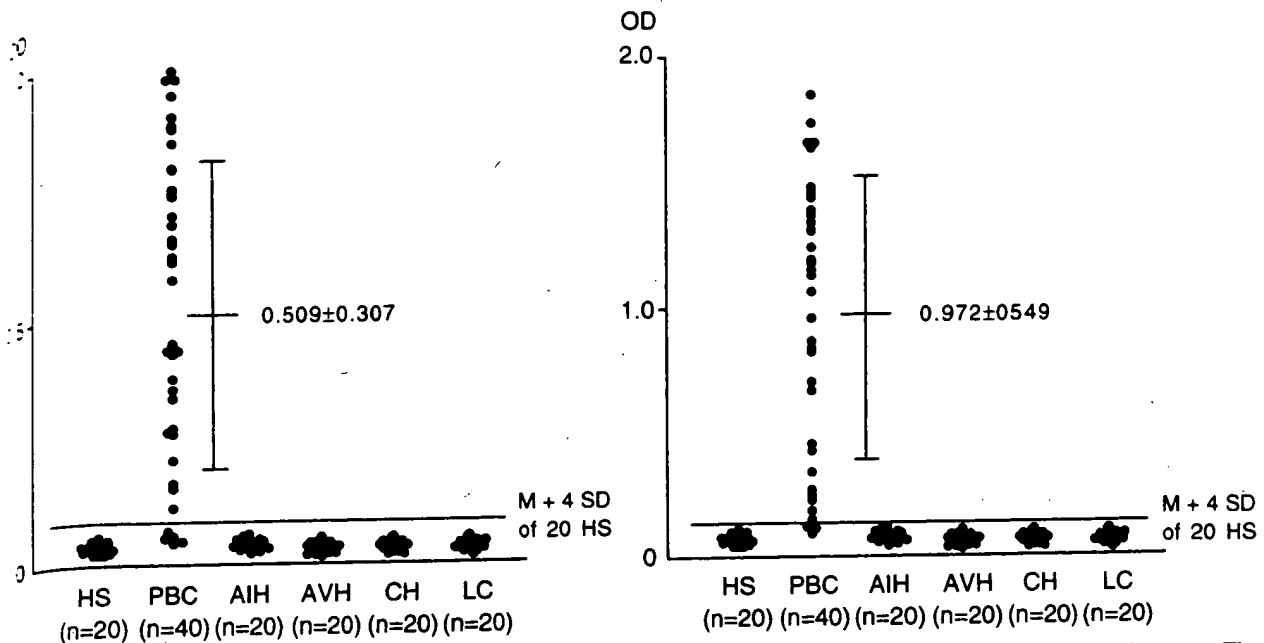


Fig. 3. The OD values on ELISA of anti-E1 and anti-E2/Pro-X antibodies in sera of patients with various liver diseases. The relative upper limit was a value +4 SD higher than the mean value of sera of 20 healthy subjects. The mean OD values of anti-E1 and anti-E2/Pro-X antibodies in 40 PBC sera were 0.509 ± 0.307 and 0.972 ± 0.549 , respectively. HS, healthy subjects ($n=20$); PBC, primary biliary cirrhosis ($n=40$); AIH, autoimmune hepatitis ($n=20$); AVH, acute viral hepatitis ($n=20$); CH, chronic hepatitis ($n=20$); LC, liver cirrhosis ($n=20$).

es (0.972 ± 0.549) of anti-E2/Pro-X antibodies in PBC sera were shown in Fig. 3. The OD values of anti-E1 antibodies correlated well with those of anti-E2/Pro-X antibodies ($r=0.918$, $p<0.01$), as seen in Fig. 4.

Effect of PBC sera on E1 activity

Table 2 shows rates of inhibition of E1 activity by 11 PBC sera as well as the OD values of anti-E1 antibodies. Serum M.H. showed the highest inhibitory rate and we therefore used this serum for the following inhibition study. $^{14}\text{CO}_2$ liberated by 30 μg of purified E1 preparation was 3072 dpm in the absence of

PBC serum. When 25, 50 and 100 μl of PBC serum M.H. were added together with purified E1, radioactivities decreased to 1937, 1116 and 796 dpm, respectively. In this assay, no inhibition was observed when we used PBC serum M.H. preabsorbed with inacti-

TABLE 2

The OD value of anti-E1 antibodies and inhibition of E1 activity by sera of PBC patients. Inhibition percentage of E1 activities was calculated as follows:

$$\% \text{ inhibition} = \left(1 - \frac{\text{dpm in presence of PBC serum}}{\text{dpm in presence of HS serum}} \right) \times 100$$

(HS: healthy subject)

Sera	OD value of anti-E1 antibodies	Inhibitory effect on E1 activity (%)
H.S.	1.04	0
AS.	0.99	8.6
E.S.	0.97	19.2
K.E.	0.93	13.4
H.T.	0.92	17.5
M.H.	0.92	63.9
Y.M.	0.75	16.9
K.H.	0.70	26.0
KK.	0.62	0
K.K.	0.46	0
I.H.	0.056	0

TABLE 1

Reaction patterns of sera of PBC patients with PDC components

Components of PDC (M.W.)					No. of positive sera (%)
E2 (4 kD)	E3 (55 kD)	Pro-X (50 kD)	E1- α (42 kD)	E1- β (37 kD)	
•		•	•	•	5 (12.5)
•		•	•		20 (50.0)
•		•			11 (27.5)
					0 (0)
Total					36 (90%)

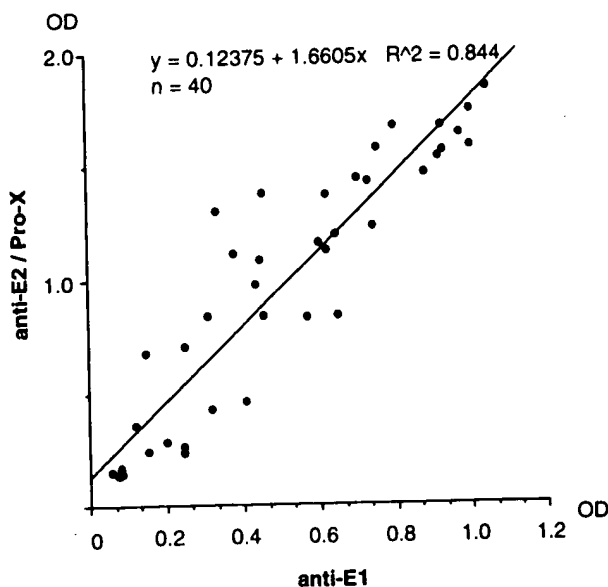


Fig. 4. Correlation between the OD values of anti-E1 and anti-E2/Pro-X antibodies in sera of 40 patients with PBC. A highly positive correlation was found between them ($r=0.918$, $p<0.01$).

vated E1 preparation, or sera of other liver disease patients and healthy subjects. This implies that anti-E1 antibodies in PBC serum M.H. bind to the active site of E1.

In Table 2, sera are arranged in order of OD values obtained for anti-E1 antibody. Serum H.S. showed the highest value at 1.04, but inhibition by this serum was 0%. In contrast, serum M.H., which showed the highest inhibitory rate of 63.9%, gave an OD value of 0.92. Thus, no positive correlation was observed between the OD value and the degree of inhibition by anti-E1 antibody. Anti-E2/Pro-X positive (anti-E1 negative) sera (I.H.) did not inhibit E1 activity at all.

Immunoglobulin classes of anti-E1 and anti-E2/Pro-X antibodies in sera of PBC patients

Values of IgG, IgM and IgA classes of anti-E1 antibodies are shown on the left side in Fig. 5. PBC sera containing anti-E1 antibodies were positive for IgG in 72.5% (29/40) of all cases, for IgM in 70% (28/40) of cases, and for IgA in 75.0% (30/40). Mean OD values of IgG, IgM and IgA antibodies in PBC sera were 0.723 ± 0.532 , 0.518 ± 0.485 and 0.257 ± 0.176 , respectively. Values of IgG, IgM and IgA classes of anti-E2/Pro-X antibodies are shown on the right side in Fig. 5. Percentages of positive reactions and mean OD values of IgG, IgM and IgA anti-E2/Pro-X antibodies in PBC sera were 85%, 0.912 ± 0.643 , 95%, 0.786 ± 0.563 and 72.5%, 0.487 ± 0.648 , respectively. Six of the 40 PBC sera were negative for IgG anti-E2/

Pro-X antibody and four of these six were positive for IgM while two of the four were also positive for IgA, as seen in Table 3. The four patients positive for IgM were all asymptomatic and histologically categorized as stage I.

When the levels of anti-E1 and anti-E2/Pro-X antibodies were compared between s-PBC and a-PBC sera, s-PBC sera showed significantly higher levels of all immunoglobulin classes (data not shown). The difference was greatest in the IgG class, as seen in Fig. 6. The mean OD values of IgG class anti-E2/Pro-X antibody of s-PBC and a-PBC sera were 1.242 ± 0.475 and 0.629 ± 0.611 , respectively.

Levels of anti-E2/Pro-X and anti-E1 antibodies of respective immunoglobulin classes were compared between PBC patients with and without other autoimmune diseases, as seen in Table 4. No significant differences were noted between them, although there was a tendency towards lower antibody levels in the PBC group manifesting other autoimmune diseases.

Discussion

Anti-PDC antibody in serum is the specific marker for PBC. Using ELISA to detect anti-PDC antibodies, it was found that the percentage of positives was 88–94% in PBC sera (6,18–20), which was higher than that of previous studies in which mitochondrial or submitochondrial fractions were used as antigens (21–23).

First, we studied antibodies against each PDC component. Even if the percentage of sera positive for anti-E1 was slightly lower than the percentage positive for anti-E2/Pro-X antibody, anti-E1 antibody could still be specifically detected in PBC sera. Levels of anti-E2/Pro-X antibody were always low in sera with low levels of anti-E1 antibody, and there were no sera containing anti-E1 antibody alone. Furthermore, when the OD values of anti-E1 and anti-E2/Pro-X antibodies were compared, there was a

TABLE 3

Serum OD values of IgM and IgA anti-E2 / Pro-X antibodies and histological stage of six patients with PBC whose sera were negative for IgG class anti-E2/Pro-X antibody

Serum of patients	IgG	IgM	IgA (OD)	Histological stage
M.O.	0.074	0.305*	0.031	I
M.S.	0.076	0.338*	0.020	I
M.K.	0.103	0.099	0.053	I
M.M.	0.101	0.185*	0.121*	I
O.S.	0.105	0.101	0.081	III
Y.S.	0.113	0.254*	0.170*	I

* Positive on ELISA.

TABLE 4
Comparison of OD values of anti-E2/Pro-X and anti-E1 antibodies of patients with or without other autoimmune diseases

Antibody	Immunoglobulin class	OD values of antibodies in patients	
		with other autoimmune disease (n=18)	without other autoimmune disease (n=22)
anti-E2/Pro-X	G	0.80±0.60	0.92±0.67
	M	0.68±0.46	0.89±0.54
	A	0.38±0.32	0.59±0.57
anti-E1	G	0.61±0.48	0.85±0.54
	M	0.54±0.44	0.64±0.46
	A	0.23±0.14	0.27±0.17

* No significance.

high positive correlation ($r=0.918$) between them. This may indicate that the immune response of PBC patients was stimulated by E2/Pro-X and E1 to a similar extent. In addition, since there were no sera reacting with E1 alone and since 11 sera reacted with only E2 and Pro-X, the epitopes to which B cells were responding might spread from E2/Pro-X to E1. In this connection, we previously reported that interleukin-6 production by peripheral blood mononuclear cells

was significantly increased by PDC stimulation of lymphocytes in PBC patients, as compared with IL-6 production in other chronic liver disease patients (24). We assumed, therefore, that in patients with PBC, PDC stimulation of lymphocytes does occur. However, the question remains as to why no anti-E3 antibodies could be detected in PBC sera. We believe that this was due to their very low titer and because we used ox E3 in this study. Using pig heart E3, Maeda et al. (25) detected anti-E3 antibodies in 11 of 29 PBC sera diluted to 1/100. When they used 1/1000 diluted sera, however, only 3 of the 29 were positive for anti-E3 antibodies and the strength of their activity was much weaker than at the 1/100 dilution. Furthermore, the E3 molecule was reported to be a weak immunogen in the presence of other components of PDC (26). In light of these findings, we believe that in PBC patients, there is a coordinated response to multiple components of PDC, indicating that the intact enzyme acts as the immunogen. With regard to T cell responses, Water et al. (27) recently reported that proliferative responses of PBMC and autoreactive T cell clones isolated from the liver of a patient with PBC against E1 and E2 components of PDC were nearly equal. In addition, they measured

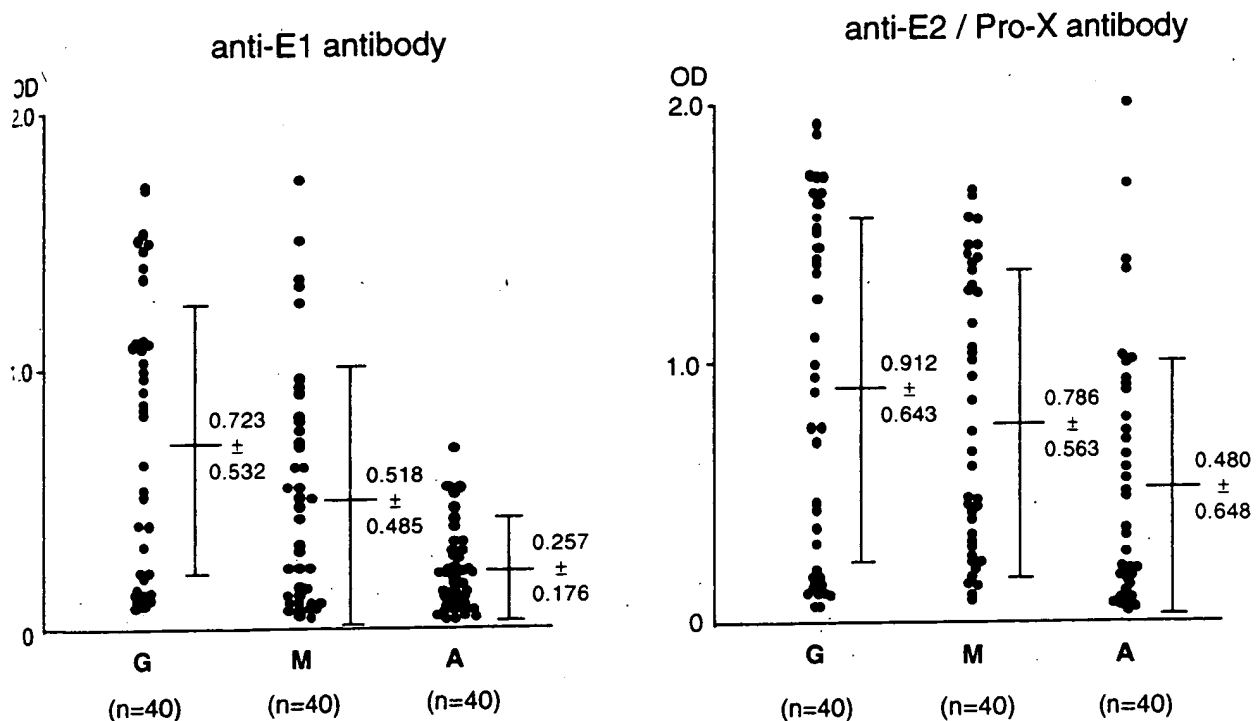


Fig. 5. OD values on ELISA of anti-E1 and anti-E2/Pro-X antibodies of IgG, IgM and IgA classes in sera of 40 PBC patients. The negative upper limit was a value +4SD higher than the mean value of sera of 20 healthy subjects. Mean OD values of IgG (G), IgM (M) and IgA (A) anti-E1 and anti-E2/Pro-X antibodies were 0.723 ± 0.532 , 0.518 ± 0.485 , 0.257 ± 0.176 and 0.912 ± 0.643 , 0.786 ± 0.563 and 0.480 ± 0.648 , respectively.

cytokine production by liver-derived E1 and E2 specific T cell clones and suggested that both T helper cell Th1- and Th2-like clones were present in the PBC liver. These Th-2-like clones induced by PDC components E1 and E2 may secrete cytokines that induce B cell-specific responses to E1 and E2. Thus, these data may also support our hypothesis that at the T cell level, an intact PDC acts as the immunogen.

It was previously reported that anti-PDC antibody in PBC patients might be derived from bacteria because of the reactivity of these sera with bacterial PDC (28). Another report has indicated that PBC patients have a higher incidence of recurrent urinary tract infection, and rough form *E. coli* were found in the urine of PBC patients with recurrent urinary infections (29). However, problems with the bacterial origin hypothesis are that bacteria do not possess the Pro-X component, although sera of PBC patients react with Pro-X, and that the antibody titer or enzyme inhibition of bacterial E2, such as that of *E. coli*, is significantly lower than against mammalian E2 (30,31). Furthermore, it was reported that antibody to E2 of mammalian PDC did not cross-react with E2 from *E. coli* (30). These reports indicate that distinct antibodies against mammalian and bacterial E2 are present in PBC sera and that lymphocytes would be more strongly stimulated by mammalian E2 than by the bacterial form. Therefore, we agree with the hypothesis proposed by Teoh et al. (31) that in

PBC patients, the initial immunogenic stimulus and antigen drive must be derived from an exogenous form of PDC and the B-cell response must then be subsequently amplified and perpetuated by the corresponding mammalian enzyme, which is either released in the course of tissue breakdown or perhaps derived from something in the meat intake of the patients. PDC is thought to be the cause of PBC or responsible for production of anti-PDC antibody, although it was reported that injection of PDC stimulated production of anti-PDC antibody in animals but did not cause a histological change in the liver (32). Recently, molecular mimicry has been considered as a possible basis for autoimmune disease. In the case of PBC, this type of mimicry has been reported to exist between the E2 component of PDC and bile duct epithelium or the α -chain of the human MHC class II antigen (33,34). In addition, MHC class II molecules have been found to be inappropriately expressed on bile duct epithelial cells in PBC patients (35,36) and we therefore propose that under such conditions, T cells induced by the PDC must react directly with these aberrantly expressed MHC class II molecules.

It was reported that affinity-purified PBC sera containing antibody to E1 inhibited the enzyme activity of PDC (7). However, there has been nothing in the literature showing inhibition by PBC sera of E1 activity or illustrating a relationship between the inhibitory rate of E1 activity and the level of anti-E1 antibody. Moreover, relationships between antibodies to each PDC component and clinical symptoms of PBC have yet to be studied in detail. In the present study, we purified PDC-E1 while maintaining its level of enzyme activity. This enabled us to carry out an ELISA of anti-E1 antibody and to measure inhibition by PBC sera of the enzyme activity of E1 itself.

It has not yet been clarified which epitopes are recognized by anti-E1 antibody or whether all PBC sera are capable of recognizing identical epitopes. In this present study, we found no correlation between the level of anti-E1 antibody and its inhibition of E1 activity. We did not titrate the antibody in the ELISA and, consequently, the affinity of our antibody was not made clear. Therefore, there might be a discrepancy between the OD value and the degree of enzyme inhibition. However, it is assumed that epitopes of E1 recognized by PBC sera would be highly varied or that affinity of the anti-E1 antibody to the epitope affected by enzyme activity might be low.

Measurement of anti-PDC antibody is important in the diagnosis of PBC, especially quantification of anti-E2/Pro-X antibodies as a means of evaluating the

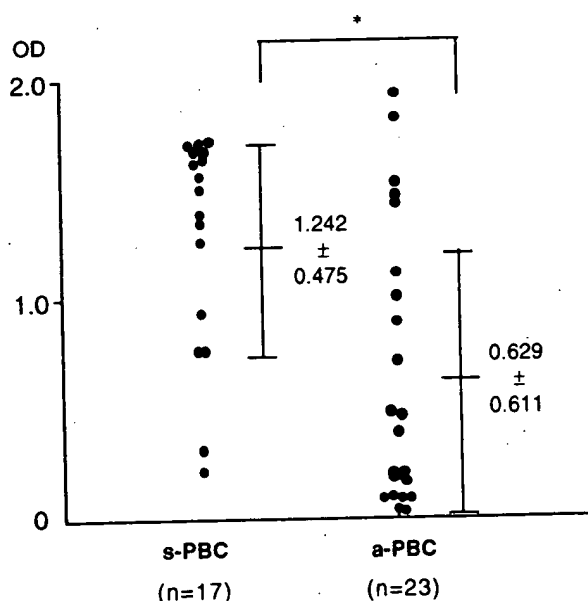


Fig. 6. Differences in serum OD values of IgG-class anti-E2/Pro-X antibodies between s-PBC and a-PBC patients (* $p < 0.05$). Mean OD values for s-PBC and a-PBC groups were 1.242 ± 0.475 and 0.629 ± 0.611 , respectively.

stimulus and an exogenous must then be by the come- h is either n or perhaps ntake of the e of PBC or DC antibody. f PDC stim- n animals but he liver (32) considered as e. In the case n reported to DC and bile human MHC IHC class II appropriately PBC patients t under such C must react MHC class II

clinical status of the PBC patients. When we measured levels of IgG class anti-E2/Pro-X antibodies, values of s-PBC patients were significantly higher than those of a-PBC patients. In the early stage of PBC, levels of IgG antibodies were low and 10% of PBC patients were negative for IgG but positive for IgM. All such patients were asymptomatic and histologically categorized as stage I. Thus, measurement of IgM class antibodies is necessary for the diagnosis of early-stage PBC. In addition, when we compared levels of anti-E1 and anti-E2/Pro-X antibodies in a group which manifested other autoimmune diseases with levels of another group which did not, we could not find any significant difference. However, there was a tendency towards lower antibody levels in the group manifesting other autoimmune diseases. Thus, we must consider the possible presence of other autoimmune diseases when we examine PBC patients with low levels of anti-PDC antibody.

In conclusion, we found that there was a high positive correlation between levels of anti-E1 and anti-E2/Pro-X antibodies. We therefore assumed that in patients with PBC, anti-PDC antibody would be produced as a result of stimulation by PDC itself. There was no relationship between the level of anti-E1 antibody and its degree of inhibition of E1 activity. Levels of IgG class anti-E2/Pro-X antibodies in s-PBC patients were significantly higher than those of a-PBC patients. Levels in the group of PBC patients manifesting other autoimmune diseases had a tendency to be lower than levels in the group which did not. In addition, 10% of PBC patients whose anti-E2/Pro-X antibodies were only of the IgM class had early-stage PBC. Thus, determination of the class of anti-E2/Pro-X antibodies is considered to be useful for the early diagnosis of PBC and for evaluation of the clinical status of the patients.

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